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## Receptor Recognition of and Immune Intracellular Pathways for *Veillonella parvula* Lipopolysaccharide<sup>▽</sup>

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*Veillonella parvula* is an anaerobic gram-negative coccus that is part of the normal flora of the animal and human mouth and gastrointestinal and genitourinary tracts. Oral *V. parvula* is involved in the development of early periodontal disease as well as different types of serious infections. Present data on molecular mechanisms responsible for innate immune response against *Veillonella* are very scanty. The aim of this study was to investigate the Toll-like receptor (TLR) pathways responsible for *V. parvula* lipopolysaccharide (LPS) and to identify the intracellular pathways induced by this recognition. *V. parvula* LPS stimulated tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) release in human peripheral blood mononuclear cells (PBMC) in a dose-dependent manner. Pretreatment of cells with a TLR4 antagonist significantly reduced TNF- $\alpha$  and IL-6 production in PBMC stimulated with either *Veillonella* or *Escherichia coli* LPS. However, *V. parvula* LPS was 10- to 100-fold less active than *E. coli* LPS for cytokine induction. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 were released in wild-type and TLR2<sup>-/-</sup>, but not TLR4<sup>-/-</sup>, mouse macrophage cultures. *V. parvula* LPS was able to activate the human PBMC p38 mitogen-activated protein kinase (MAPK). A specific p38 MAPK inhibitor strongly inhibited *V. parvula* LPS-induced TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10. In conclusion, *V. parvula* LPS is able to induce cytokine production in both human and murine in vitro models, although it is less effective than *Enterobacteriaceae* LPS. *V. parvula* LPS-stimulated cytokine induction, as well as p38 MAPK activation, are TLR4-dependent features.

*Veillonella* organisms are small, nonfermentative, strictly anaerobic, gram-negative cocci which form part of the normal flora of the oral, genitourinary, respiratory, and intestinal tracts of humans and animals (10). The genus *Veillonella* was first isolated by Veillon and Zuber in 1898 and currently consists of eight species (28).

*Veillonella* species have been reported as causes of serious infections, including meningitis (6), osteomyelitis and discitis (7, 28), prosthetic joint infection (26), and acute and chronic pleuropulmonary infection (33).

Risk factors for *Veillonella* infection include periodontal disease, immunodeficiency, intravenous drug use, and premature birth (28). *V. parvula* is an important pathogen implicated in periodontitis and other dental infections (3, 18), and it is one of the most common anaerobic pathogens in chronic maxillary sinusitis and deep neck infections (9, 37). *V. parvula* has also been reported as a pathogen for osteomyelitis (34) and abscessed orchiepididymitis with sepsis (4). Endovascular infections reportedly may range from bacteremia to severe endocarditis and fatal cases of sepsis (8, 14, 25).

Lipopolysaccharides (LPS) are major pathogenic factors of gram-negative bacteria. LPS from aerobic and facultative bacteria have been extensively studied (5). On the contrary, very

little is known regarding the biological activity of LPS from anaerobic microorganisms such as *Veillonella* (10, 24, 29, 32). In addition, little is known about cellular and molecular mechanisms responsible for innate immune response against *V. parvula*, as well as for inflammatory reactions leading to severe periodontitis or sinusitis. Toll-like receptors (TLRs) recognize microbial compounds (36) and trigger the inflammatory and immune responses against pathogens. Immunohistochemical localization of TLR2 and TLR4 in gingival tissue of periodontitis patients has been reported (30). In mammals, engagement of TLRs by LPS results in the recruitment of cytoplasmic signaling molecules (12, 36), which eventually activates mitogen-activated protein kinases (MAPKs), including p38, JNK, and extracellular signal-regulated kinase (ERK). MAPK activation leads to cytokine release (2).

However, the interaction between oral *V. parvula* LPS and TLRs has not been directly studied yet. The aim of this study was to investigate the potential role of TLR2 and TLR4 for the recognition of *Veillonella parvula* LPS in both human peripheral blood mononuclear cells (PBMC) and in TLR2 and TLR4 knockout (KO) mouse macrophages, as well as the intracellular kinase signaling pathways induced after challenge of monocytes with *Veillonella parvula* LPS.

### MATERIALS AND METHODS

***Veillonella parvula* culture and LPS purification.** *V. parvula* ATCC 10790 (American Type Culture Collection, Rockville, MD) was grown anaerobically in modified lactate broth at 37°C. After *V. parvula* reached the early stationary phase (about 40 h of incubation), bacterial cells were harvested by centrifugation

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and washed twice in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM 2-mercaptoethanol (PPB). Cells were extracted twice with phenol-water (38). Briefly the aqueous layers were combined and dialyzed against 20 liters of distilled water at 4°C. The crude LPS was lyophilized and then dissolved in distilled water and centrifuged once at  $80,000 \times g$  for 7 h at 4°C. The pellet was suspended in distilled water and recentrifuged twice at  $105,000 \times g$  for 3 h at 4°C. The LPS was dissolved in PPB with RNase (Sigma, Chemical Co., St. Louis, MO) and DNase (Sigma) at 20  $\mu\text{g}/\text{ml}$  each. After 2 h of incubation at 37°C, the solution was centrifuged at  $105,000 \times g$  for 3 h at 4°C. The pellet was washed in distilled water and centrifuged twice at  $105,000 \times g$  at 4°C. The final pellet was dissolved in distilled water, lyophilized, and stored at  $-20^\circ\text{C}$  as once-purified LPS. Double-step-purified LPS (repurified LPS) was obtained following the method described by Hirschfeld et al. (17).

**Isolation of peripheral blood mononuclear cells and stimulation of cytokine production.** Isolation of PBMC was performed as described elsewhere (1), with minor modifications. All volunteers signed an informed consent form, according to the institutional guidelines and procedures. A total of  $5 \times 10^5$  PBMC in a 100- $\mu\text{l}$  volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100  $\mu\text{l}$  of culture medium or the various stimuli: highly purified *Veillonella* LPS (1 to 100 ng/ml), highly purified *E. coli* LPS (1 ng/ml; Sigma), or Pam<sub>3</sub>Cys (10  $\mu\text{g}/\text{ml}$ ; EMC Microcollections). In some experimental sets, cells were pretreated with or without double-extracted *Bartonella quintana* LPS (1  $\mu\text{g}/\text{ml}$ ), a TLR4 antagonist (31), 30 min before treatment with *V. parvula* LPS (100 ng/ml). Specific signal transduction inhibitors of p38 MAPK (SB202190; 25  $\mu\text{M}$ ), ERK1/2 (U0126; 25  $\mu\text{M}$ ), JNK1/2/3 kinase (SP600125; 25  $\mu\text{M}$ ) (all inhibitors purchased from Superarray Bioscience Corporation, Bethesda, MD) were added to the cultures for 45 min prior to addition of *Veillonella* LPS. All unstimulated and control samples received an equal amount of inhibitor solvent, dimethyl sulfoxide (0.1% [vol/vol]). After 24 h of culture, the supernatants were collected, centrifuged at 1,200 rpm, and stored at  $-80^\circ\text{C}$  until assayed.

**Isolation of peritoneal macrophages and stimulation of cytokine production.** For isolation of mouse peritoneal macrophages and spleen cells, groups of five TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice (kindly provided by S. Akira, Osaka, Japan) and their control littermates were sacrificed, and resident peritoneal macrophages were harvested by injecting 4 ml of sterile phosphate-buffered saline containing 0.38% sodium citrate. After washing, the cells were resuspended in RPMI 1640 medium containing 1 mM pyruvate, 2 mM L-glutamine, 100  $\mu\text{g}/\text{ml}$  gentamicin, and 2% fresh mouse plasma (culture medium). Cells were cultured in 96-well microtiter plates (Greiner, Alphen, The Netherlands) at  $10^5$  cells per well, in a final volume of 200  $\mu\text{l}$ . The cells were stimulated with either control medium or highly purified *Veillonella* LPS (1  $\mu\text{g}/\text{ml}$ ), highly purified *E. coli* LPS (1 ng/ml), or Pam<sub>3</sub>Cys (10 ng/ml). After 24 h of incubation at 37°C, the plates were centrifuged ( $500 \times g$ , 10 min), and the supernatant and cell lysate (three freeze-thaw cycles) were collected and stored at  $-80^\circ\text{C}$  until cytokine assays were performed.

**Cytokine determinations.** Human tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, and IL-10 were measured by using either specific or commercial enzyme-linked immunosorbent assay (ELISA) kits (from R&D Systems, Abingdon, United Kingdom, and the Pelikine Compact from Sanquin, Amsterdam, The Netherlands). Sensitivities of both ELISAs were below 5 pg/ml (13). Murine IL-1 $\alpha$  and TNF- $\alpha$  were measured with a radioimmunoassay, and murine IL-6 and IL-10 were determined using ELISA kits (R&D Systems, Abingdon, United Kingdom).

**Western blot analysis of *Veillonella* LPS signal transduction.** After the isolation of human PBMC, aliquots of the cells were placed in 3-cm sterile tissue culture plates in 1 ml of RPMI 1640 medium complemented with RPMI 1640 containing 10% fetal bovine serum, 10 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA). The cells were immediately pretreated with or without the TLR4 antagonist *B. quintana* LPS (1  $\mu\text{g}/\text{ml}$ ) (31) added 30 min before treatment with *V. parvula* LPS (100 ng/ml).

At different time points the cells were collected and the proteins were extracted using a lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA (pH 8), 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.5% NP-40, 10% glycerol, 0.5 mM dithiothreitol, 10 mM NaVO<sub>3</sub>, 100 mM NaF, 100  $\mu\text{g}/\text{ml}$  leupeptin, 0.5 mM phenylmethylsulfonyl fluoride. After incubation for 30 min on ice, lysates were centrifuged at  $10,000 \times g$  for 10 min at 4°C. A 30- $\mu\text{g}$  portion of cell extract was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were incubated with anti-p38, anti-phospho-p38, anti-ERK, anti-phospho-ERK, anti-JNK, or anti-phospho-JNK (1:1,000; Cell Signaling), or anti-actin (1:200; Santa Cruz) antibodies and then with anti-rabbit or anti-mouse horseradish peroxidase-coupled secondary antibody (Amersham, Buckinghamshire, United Kingdom). Immune com-

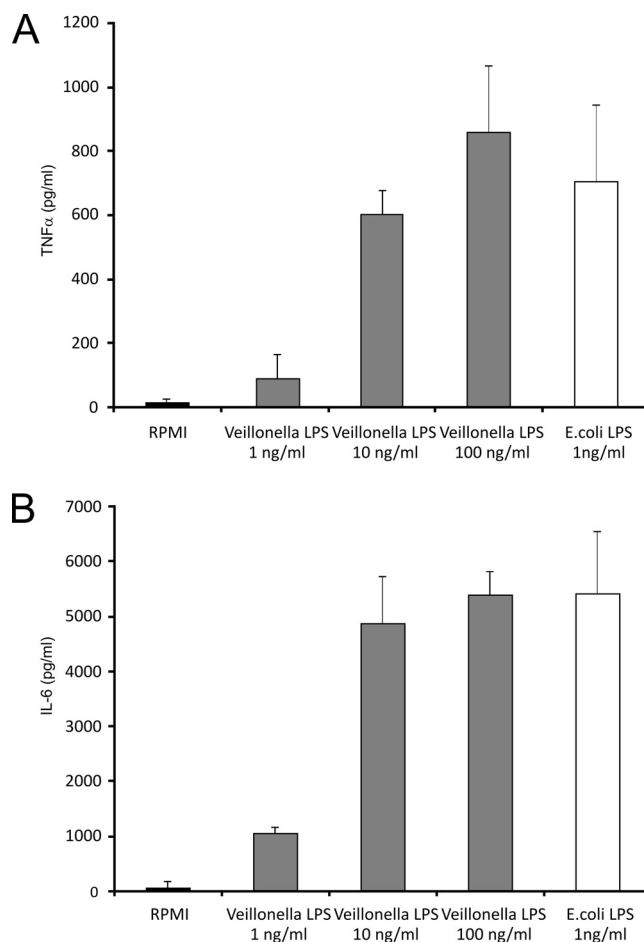


FIG. 1. (A) TNF- $\alpha$  production after exposure to *Veillonella* LPS. Human PBMC were stimulated with a dose of purified *Veillonella* LPS for 24 h. *E. coli* LPS served as a positive control. Data represent the means  $\pm$  standard deviations for cytokine production by at least four healthy volunteers. (B) IL-6 production. Cytokine levels were determined by an ELISA, and the detection limit was 20 pg/ml for both cytokines.

plexes were identified by using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

**Statistical analysis.** The data are expressed as mean  $\pm$  standard errors of the means unless indicated otherwise. Differences between experimental groups were tested using the Mann-Whitney U-test, performed using GraphPad Prism 4.0 software. *P* values of 0.05 or less were considered significant.

## RESULTS

***V. parvula* LPS is a weak stimulator of cytokine production in human PBMC.** Following stimulation with increasing amounts of *V. parvula* LPS (1 to 100 ng/ml), human PBMC released TNF- $\alpha$  and IL-6 in a dose-related fashion. The highest concentration of *V. parvula* LPS used (100 ng/ml) released an amount of the above cytokines that was comparable to that produced by 1 ng/ml of the reference *E. coli* LPS (Fig. 1A and B).

**A TLR4 antagonist blocks *V. parvula* LPS-induced cytokine production.** When human PBMC were pretreated with a TLR4 antagonist (*B. quintana* LPS at 1  $\mu\text{g}/\text{ml}$ ), the release of TNF- $\alpha$  and IL-6 following stimulation with *V. parvula* LPS (1 to 100

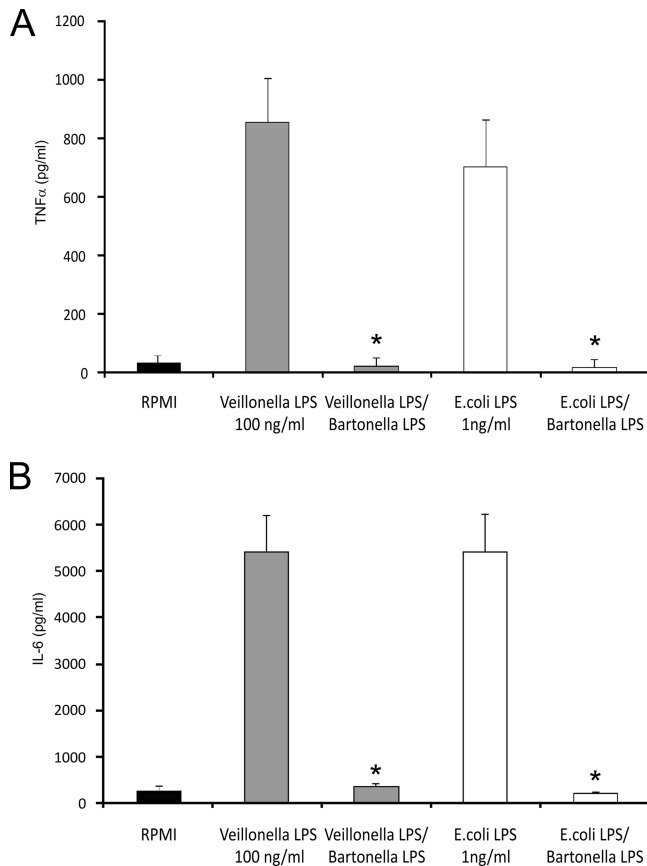


FIG. 2. Inhibition of *Veillonella* LPS-induced TNF- $\alpha$  (A) and IL-6 (B) production by a TLR4 antagonist. PBMC were pretreated with *Bartonella quintana* LPS for 1 h and then either *Veillonella* LPS or *E. coli* LPS was added. Data represent the means  $\pm$  standard deviations for cytokine production of at least four healthy volunteers. Note the complete suppression of cytokine production after TLR4 blockade. \*,  $P < 0.01$  (Wilcoxon rank test).

ng/ml), as well as with 1 ng/ml of the reference *E. coli* LPS, was strongly inhibited (Fig. 2A and B).

**TLR4 is the major receptor for *V. parvula* LPS-induced cytokine production in mice.** Resident peritoneal macrophages were harvested from TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice and their control littermates and stimulated in vitro with either control medium or highly purified *Veillonella* LPS (1  $\mu$ g/ml), highly purified *E. coli* LPS (1 ng/ml), or Pam<sub>3</sub>Cys (10 ng/ml). The three stimuli used were able to produce a cytokine release substantially higher than medium alone in control macrophage cultures. Although *Veillonella* LPS caused a significant release of cytokines in the macrophages from TLR2<sup>-/-</sup> mice, it was not able to induce a relevant release of cytokines among the macrophages from TLR4<sup>-/-</sup> mice (Fig. 3A and B and 4A and B).

***Veillonella*-induced activation of p38 MAPK, which mediates cytokine production.** *V. parvula* LPS was able to activate the human PBMC p38 MAPK after 60 minutes of incubation. When *B. quintana* double-purified LPS was used to inhibit TLR4 on PBMC, activation of p38 MAPK was hardly inhibited after 60 minutes (Fig. 5A).

The activation of JNK and ERK1/2 after the *V. parvula* LPS stimulus was very inconsistently observed regardless of the

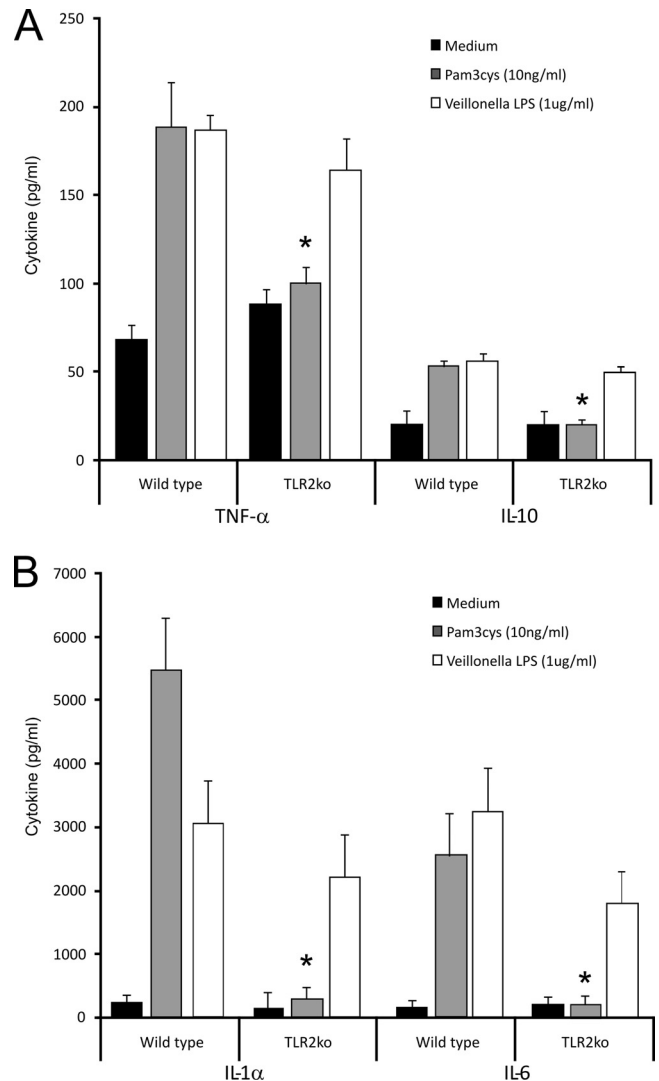


FIG. 3. TLR2 is not activated by *Veillonella* LPS. Peritoneal macrophages from wild-type and TLR2 gene-deficient mice were stimulated with *Veillonella* LPS for 24 h. As a control, cells were stimulated with Pam<sub>3</sub>Cys (a potent TLR2 ligand). (A) Murine TNF- $\alpha$  and IL-10 production induced by *Veillonella* LPS. (B) Murine IL-1 $\alpha$  and IL-6 levels. Note that Pam<sub>3</sub>Cys-induced cytokine production was abrogated in TLR2 knockout (TLR2ko) cells. Data represent the means  $\pm$  standard deviations for cytokine production by at least five mice. \*,  $P < 0.01$  (Wilcoxon rank test).

time point considered (15, 30, and 60 min) (data not shown). In line with these data, a p38 MAPK inhibitor strongly reduced cytokine production induced by *V. parvula* LPS (Fig. 5B and C). Similar results were observed with an inhibitor of JNK kinase, while inhibition of ERK had a more modest effect (Fig. 5B and C).

## DISCUSSION

In the present paper we demonstrate that *V. parvula* LPS is recognized by TLR4 in both humans and mice and that the activation of MAP kinases plays an important role in *V. parvula* LPS-stimulated human PBMC signaling.

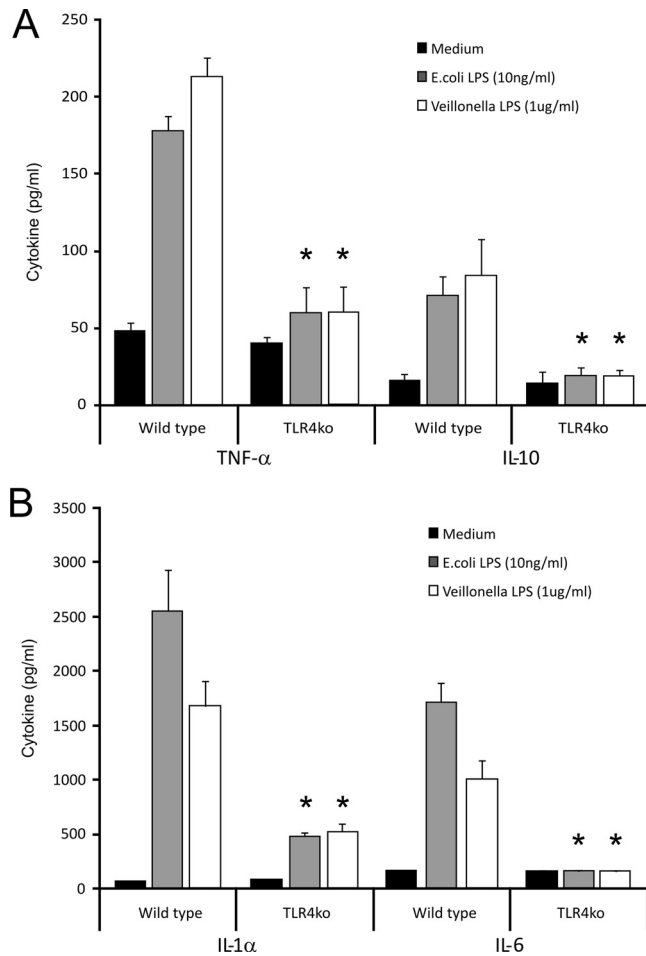


FIG. 4. *Veillonella* LPS-induced cytokine production is TLR4 dependent. Peritoneal macrophages from wild-type and TLR4 gene-deficient mice were stimulated with *Veillonella* LPS for 24 h. As a control, cells were stimulated with *E. coli* LPS (a potent TLR4 ligand). (A) Murine TNF- $\alpha$  and IL-10 production induced by *Veillonella* LPS. (B) Murine IL-1 $\alpha$  and IL-6. Note that both *Veillonella* LPS and *E. coli* LPS induction of cytokine production was abrogated in TLR4 knock-out (ko) cells. Data represent the means  $\pm$  standard deviations for cytokine production by at least five mice. \*,  $P < 0.01$  (Wilcoxon rank test).

Previous studies on the in vitro and in vivo activities of the LPS from *Veillonella parvula* demonstrated that such an endotoxin has biological effects comparable to enterobacterial LPS (10, 29). Earlier studies reported on fibroblast collagenase-inducing PBMC cytokines stimulated by *Veillonella* spp. LPS; however, such cytokines were not directly evaluated (16). Formalin-killed *V. parvula* has been demonstrated to induce the release of IL-1 $\beta$  in human blood monocytes at levels comparable to those released by *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Prevotella intermedia* (27). More recent investigations showed that the concentrations of TNF- $\alpha$ , IL-6, and IL-10 released from umbilical cord and adult mononuclear cells following in vitro challenge with UV-killed *V. parvula* were comparable or even higher than cytokine concentrations evaluated in *Pseudomonas aeruginosa*- and *E. coli*-stimulated cord and adult cells (19).

The lower cytokine production of PBMC induced by *Veil-*

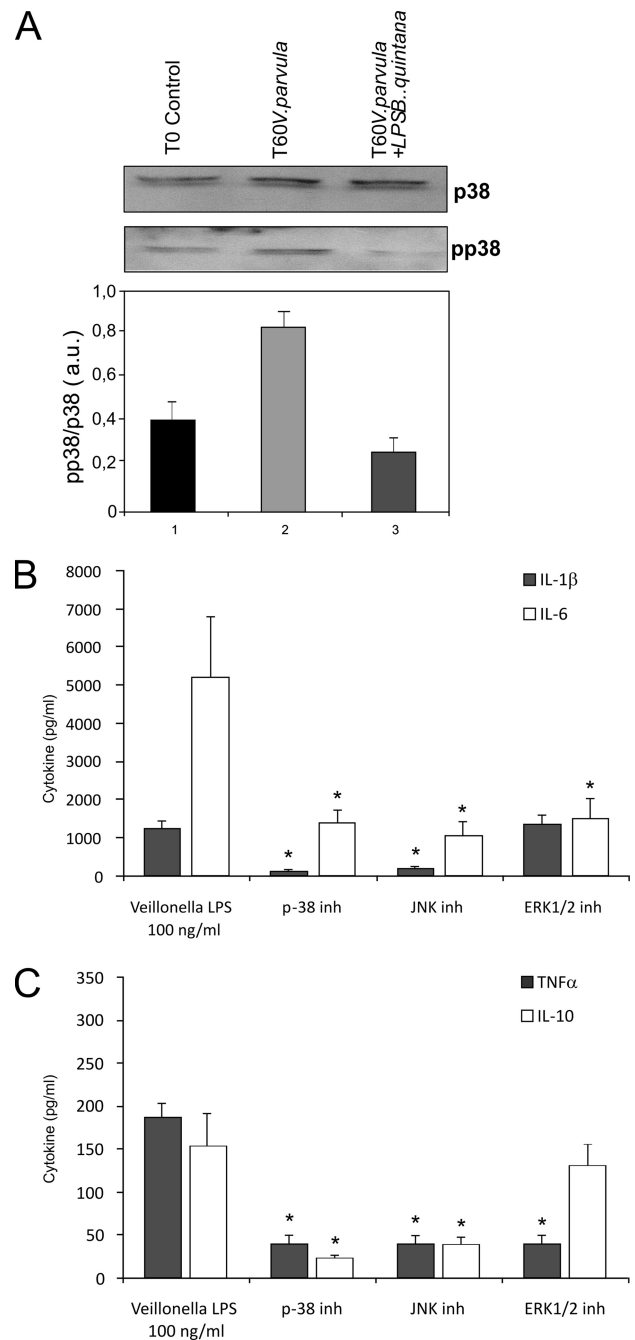


FIG. 5. *Veillonella* LPS activates p38 MAPK. (A) Effects of TLR4 inhibition by *B. quintana* LPS on *Veillonella* LPS-stimulated p38 activation in human PBMC. Protein extracts (30  $\mu$ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting using specific anti-p38 and anti-phospho-p38 antibodies. The intensity of phospho-p38-specific bands was measured by densitometry and is reported as arbitrary units (a.u.) of phospho-p38 expression after normalization with p38 levels. The means  $\pm$  standard errors of the means of three independent experiments are shown. (B and C) Effects of pharmacological inhibition of p38 MAPK-, JNK-, and ERK-mediated pathways during *Veillonella* LPS stimulation of PBMC; the graphs show IL-1 $\beta$  and IL-6 production after inhibition of p38 MAPK, JNK, and ERK (B) or TNF- $\alpha$  and IL-10 production (C). Data represent the means  $\pm$  standard deviations for cytokine production of at least four healthy volunteers. Note the strong suppression of cytokine production after inhibition of p38 MAPK and JNK. \*,  $P < 0.01$  (Wilcoxon rank test).



*Veillonella* LPS (approximately 20 to 30% of that induced by *E. coli* LPS) is mirrored in the type of pathology induced by these microorganisms. On the one hand, gram-negative sepsis with enterobacteriaceae induces a superacute inflammatory reaction, followed sometimes by organ failure and even death. On the other hand, *Veillonella* is present in the mouth flora and is an important pathogen of periodontal disease, a chronic inflammatory condition. Thus, the low cytokine production by *Veillonella* is certainly important in inducing the low-grade inflammation in periodontitis.

To the best of our knowledge, very few papers have reported on the role of TLRs for the pathogenesis of *Veillonella* infection. In one study treatment of dendritic cells with an anti-TLR4 antibody decreased cytokine production induced by UV-treated *V. parvula* (20), but the nature of the *Veillonella* ligand recognized by TLR4 was not identified.

Similarly, TLR2- and TLR4-transfected human embryonic kidney cells responded to sonicated *Veillonella* bacteria stimulation (21). In both these studies, *Veillonella* seemed to stimulate both TLR4 and TLR2. However, the bacterial product responsible for the TLR engagement was not addressed, and the models used were only from murine species. In the present study, we demonstrate that *V. parvula* LPS is recognized by TLR4 in both human and murine cells, and we show the MAPK role in *Veillonella* LPS-stimulated human PBMC signaling.

*V. parvula* has been implicated in both dental/periodontal diseases (3, 18) and joint disorders (26). Periodontal disease and rheumatoid arthritis have remarkably similar inflammatory mediator profiles (22). Within periodontal lesions, activated monocytes, macrophages, and fibroblasts produce cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, which have been found to be significantly elevated in diseased periodontal sites compared with healthy or inactive sites (11). These cytokines orchestrate the cascade of destructive events that occur in the periodontal tissues and trigger the production of an array of inflammatory enzymes and mediators, resulting in irreversible hard and soft tissue damage (15). By exploiting an in vitro model of periodontal diseases, it has been found that upregulation of IL-6 by lipopolysaccharide treatment is TLR4 dependent. This pattern of gene expression indicates that pathogens may trigger TLR4 signaling and cause periodontitis (35). Therefore, our present findings may have sound clinical impact. Moreover, due to the similarity of pathogenesis between periodontitis and rheumatoid arthritis, antagonists of TLR4, as well as p38 inhibitors, have the potential to ameliorate progression of periodontal disease (23), rheumatic pathologies, and other chronic inflammatory/degenerative disorders. The TLR4 antagonist *B. quintana* LPS (31) has been demonstrated to dramatically improve the evolution of experimental arthritis in the mouse (1). Further studies are warranted in order to exploit the modulation of TLR4 and p38 MAPK in the therapy of chronic inflammatory/degenerative diseases.

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